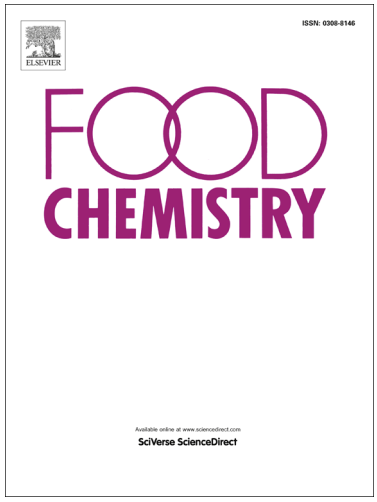

Analytical Methods

Determination of aflatoxin M1 in milk samples by means of an inductively coupled plasma mass spectrometry-based immunoassay

Emma Pérez, Pascual Martínez-Peinado, Francisco Marco, Luis Gras, José Miguel Sempere, Juan Mora, Guillermo Grindlay

PII: S0308-8146(17)30455-7
DOI: http://dx.doi.org/10.1016/j.foodchem.2017.03.078
Reference: FOCH 20782

To appear in: *Food Chemistry*

Received Date: 3 March 2016
Revised Date: 24 January 2017
Accepted Date: 13 March 2017

Please cite this article as: Pérez, E., Martínez-Peinado, P., Marco, F., Gras, L., Sempere, J.M., Mora, J., Grindlay, G., Determination of aflatoxin M1 in milk samples by means of an inductively coupled plasma mass spectrometry-based immunoassay, *Food Chemistry* (2017), doi: http://dx.doi.org/10.1016/j.foodchem.2017.03.078

Determination of aflatoxin M1 in milk samples by means of an inductively coupled plasma mass spectrometry-based immunoassay

Emma Pérez[#], Pascual Martínez-Peinado[&], Francisco Marco[&], Luis Gras[#], José Miguel Sempere[&], Juan Mora[#], Guillermo Grindlay[#*]

[#] *Department of Analytical Chemistry, Nutrition and Food Sciences, University of Alicante. PO Box 99, 03080 – Alicante, Spain.*

[$] *Department of Biotechnology, University of Alicante. PO Box 99, 03080 – Alicante, Spain.*

*corresponding author e-mail address: guillermo.grindlay@ua.es

Abstract

An inductively coupled plasma mass spectrometry (ICP-MS)-based immunoassay has been developed to quantify aflatoxin M1 (AFM1) at ultra-trace levels in milk samples. AFM1 detection is carried out by means of a competitive immunoassay using secondary biotinylated antibodies and streptavidin-conjugated Au nanoparticles. After acid addition, nanoparticles are decomposed and Au signal is registered by means of ICP-MS. Results demonstrate that, under optimum conditions, the limit of detection of the immunoassay (0.005 μg kg^{-1}) is low enough to quantify AFM1 according to current international policies (including the more restrictive European one). Method accuracy and precision was checked by analyzing an AFM1 certified reference material and different milk samples spiked with known amounts of AFM1. AFM1 recovery values range from 80% to 102% whereas inter-assay and intra-assay precision are lower than 15%. Finally, this immunoassay methodology affords a higher dynamic working range (0.012-2.5 μg kg^{-1}) than other immunoassay methodologies described in the literature.

Keywords: aflatoxin M1, milk, immunoassay, gold nanoparticles, inductively coupled plasma mass spectrometry

1. Introduction

Aflatoxins are secondary metabolites produced by different filamentous fungi (mainly *Aspergillus* species) and they are known to represent a high risk for human health due to their mutagenic and teratogenic effects. These substances could be found in different kinds of foods and animal feeds (e.g. cereals, cocoa, coffee, etc.) that have been in contact with fungi through the food chain under high temperature and humidity conditions (Bhat, Rai, & Karim, 2010).

Aflatoxin M1 (AFM1) is the most significant aflatoxin in milk and dairy products. This compound is the hydroxylated form of the aflatoxin B1 (AFB1) and it is usually present in milk when animals have been fed with feedstuffs containing AFB1 (Flor-Flores, Lizarraga, López de Cerain, & González-Peñas, 2015). AFM1 has been classified as Group 2 human carcinogen by the International Agency of Research on Cancer (IARC, 2002). For this reason, and taking into account the significance of milk and milk products in human diet (especially for children), the maximum allowed levels of AFM1 are strictly regulated worldwide (Flor-Flores, Lizarraga, López de Cerain, & González-Peñas, 2015; Prandini, Tansini, Sigolo, Filippi, Laporta, & Piva, 2009). Food and Drug Administration from USA limits the concentration of AFM1 in milk and processed milk products at 0.50 μg kg^{-1} (FDA, 2005). However, European Community Legislation is even more restrictive and does not allow AFM1 levels in milk and infant formula above 0.050 and 0.025 μg kg^{-1}, respectively (EC, 2002; EC, 2004).

AFM1 determination is usually carried out by means of high-performance liquid chromatography (HPLC) or immunoassays after an extraction treatment to reduce matrix effects and pre-concentrate the analyte (Shephard, 2008; Reiter, Zentek, & Razzazi, 2009). HPLC is considered the reference method for AFM1 analysis (Dragacci, Grosso, & Gilbert, 2001; Diniz Andrade, Laine Gomez da Silva, & Dutra Caldas, 2013; Aguilera-Luiz, Plaza-Bolaños, Romero-González, Martínez Vidal, & Garrido Frenich, 2011; Beltran, Ibañez, Sancho, Cortés, Yusa, & Hernández, 2011). The detection of AFM1 is generally achieved by means of both fluorescence and mass spectrometry. However, HPLC analysis requires laborious sample preparation treatments to reduce matrix effects and improve analytical figures of merit. On the other hand, immunoassays (mainly Enzyme Linked Immunosorbent Assay, i.e., ELISA) are widely used for screening purposes due to their high sample throughput, simplicity and low budget (Picó, 2007; Guan, Li, Zhang, Zhang, & Jiang, 2011; Jiang, Wang, Nolke, Zhang, Niu, & Shen, 2013; Vdovenko, Lu, Yu, Sakharov, 2014).

Inductively coupled plasma mass spectrometry (ICP-MS) is a powerful technique for inorganic analysis due to its: (i) low limits of detection (LoD) (usually in the μg kg^{-1}-ng kg^{-1} range), (ii) good precision; (iii) multi-element capability, (iv) high dynamic range; and (v) the possibility to obtain analyte isotopic information (Bettmer, Montes-Bayón, Ruiz Encinar, Fernández, Fernández de la Campa, & Sanz-Medel, 2009). Traditionally, the analysis of organic molecules by ICP-MS has been limited to those analytes containing metals, metalloids and some non-metals (e.g. P or S) because of the difficulty to utilize C, H, N and O for quantification purposes at ultra-trace levels. However, it has been demonstrated that ICP-MS can be used as a detector for all kinds of

organic molecules (e.g. proteins, mRNA, DNA, etc.) after a derivatization procedure with a heteroatom or a compound containing a heteroatom (Tholey & Schaumlöffel, 2009; Kretschy, Koellensperger, & Hann, 2012). In this context, ICP-MS has been employed as a detector of proteins and biomolecules in immunoassays in view of it is quite straightforward to functionalized antibodies with elements detectable by this technique (Giesen, Waentig, Panne, & Jakubowski, 2012; Liu, Wu, Yang, Hou, & Lv, 2014). In general, antibodies (or any other species present in the immunoassay) are conjugated with elements presumably not present in biological samples, such as lanthanide based chelates or Au nanoparticles. The latter approach is especially advantageous to amplify the analytical response because of the significant number of quantities of Au atoms in each nanoparticle. The use of ICP-MS as a detector in immunoassays affords several attractive features such as: (i) specificity to heteroatom detection; (ii) compound-independent detection sensitivity; (iii) high elemental sensitivity and dynamic range; (iv) robustness (complex sample pre-treatments are not required to diminish matrix effects); and (vii) multiplexing capabilities, since the antibodies can be conjugated with different heteroatoms and detected simultaneously. In spite of the above mentioned features, the use of ICP-MS-based immunoassays in food analysis has been limited so far. Nonetheless, these methods have been successfully applied to quantify peanut allergens (Careri, Elviri, Mangia, & Mucchino, 2007), ochratoxine A in wine (Giesen, Jakubowski, Panne, & Weller, 2010) and progesterone in milk (Montoro Bustos, Trapiella-Alfonso, Ruiz Encina, Costa-Ferández, Pereiro, & Sanz-Medel, 2012).

The goal of this work is to develop a new procedure to quantify AFM1 in milk samples by ICP-MS at the security levels required by the current international policies with accuracy and precision. The proposed methodology is based on a competitive immunoassay using secondary biotinylated antibodies and streptavidin-Au nanoparticles followed by Au detection by ICP-MS.

2. Experimental

2.1. Reagents and materials

All solutions were prepared using ultrapure water (Milli-Q water purification system, Millipore Inc., Paris, France).

Sodium carbonate, sodium hydrogen carbonate, monosodium phosphate, disodium phosphate, sodium chloride, biotinylated goat anti-rabbit IgG secondary antibody, AFM1 from Aspergillus flavus, AFM1-Bovine Serum Albumin conjugate (AFM1-BSA), streptavidin conjugated 40 nm Au nanoparticles from *Streptomyces avidinii*, polyethylene glycol sorbitan monolaurate (Tween 20) and HPLC-grade acetonitrile were purchased from Sigma-Aldrich (Steinheim, Germany). Bovine serum albumin (BSA) was obtained from Biowest (Nuaillé, France) whereas anti-AFM1 primary rabbit polyclonal antibody was obtained from Agrisera (Vännas, Sweeden). Iridium 1000 mg L^{-1} stock solution was provided by Merck (Darmstadt, Germany). Thiourea, 69% w w^{-1} nitric acid and 35% w w^{-1} hydrochloric acid were purchased from Panreac (Barcelona, Spain).

F16 maxisorp polystyrene microtiter plates were obtained from Thermo-Scientific (Roskilde, Denmark).

2.2. Buffers and solutions

Standard stock solution of AFM1 (10 µg L^{-1}) was prepared in pure acetonitrile in an amber vial. AFM1-BSA was dissolved in 2 mL of phosphate buffer solution (PBS, 10 mol L^{-1} monosodium phosphate, 2 mmol L^{-1} disodium phosphate, 154 mmol L^{-1} sodium chloride, pH 7.6) for a final concentration of 500 µg mL^{-1}. Both solutions were kept at -20ºC. Primary rabbit polyclonal antibody was dissolved in 500 µL ultrapure water and kept at 4ºC.

The following solutions were employed in the ICP-MS-based immunoassay: (a) carbonate/bicarbonate buffer solution (15 mmol L^{-1} sodium carbonate and 35 mmol L^{-1} sodium hydrogen carbonate, pH 9.6); (b) 1% w V^{-1} BSA in a PBS solution for plate blocking; (c) 1% w V^{-1} BSA and 0.05% V V^{-1} Tween 20 in PBS as the incubation media for the antibodies and streptavidin-Au (Strep-Au) media; (d) 0.05% V V^{-1} Tween 20 in PBS for washing microtiter plate wells and (d) 4% V V^{-1} nitric acid and 12% V V^{-1} hydrochloric acid for Au-nanoparticles digestion.

2.3. Immunoassay procedure

The analysis of AFM1 by ICP-MS is based on a competitive immunoassay (Giesen, Jakubowski, Panne, & Weller, 2010) in which varying amounts of free AFM1 inhibit the binding of specific antibodies to the solid phase coated with AFM1-BSA conjugate using secondary biotinylated antibodies and Strep-Au for ICP-MS detection (see Figure 1). First of all, the polystyrene microtiter plate wells were coated with 100 µL of the appropriate AFM1-BSA concentration in carbonate-bicarbonate buffer (step 1a). After a 1 hour incubation at room temperature, wells were washed 3 times and blocked with 1% w V^{-1} BSA in

PBS for 1 hour at room temperature. Simultaneously, samples or AFM1 standards were mixed with the anti-AFM1 antibody solution (step 1b). The mixture was incubated 1 hour at room temperature and then 100 µL of it were transferred to the plate wells for another incubation step of 2.5 hours at room temperature (step 2). After washing -three times in order to eliminate the antigen-antibody complexes present in the solution as well as the free antibody, the microwell plates were sequentially incubated with 100 µL of a secondary biotinylated goat anti-rabbit IgG solution (step 3) and then with 100 µL of the Strep-Au solution (step 4). The incubation time for the previous steps was 1 hour at room temperature followed by three washing steps. Finally, before ICP-MS analysis, Au nanoparticles were digested (step 5) with 150 µL of the digestion acid mixture and spiked with 50 µL of a 1.0% w V^{-1} thiourea solution containing 2.5 µg L^{-1} Ir. All the AFM1 standards were analyzed in triplicate wells whereas samples containing unknown AFM1 amounts in quintuplicate wells.

2.4. ICP-MS instrumentation

According to the immunoassay procedure described above, AFM1 is quantified by means of ICP-MS using the $^{197}Au^{+}$ signal. Despite the poor Au ionization in the plasma because of its high ionization potential (9.23 eV) (NIST atomic spectradatabase, 2016), the use of Au nanoparticles is especially advantageous to amplify the analytical response due to the high number of atoms present in each nanoparticle. ICP-MS measurements were performed by means of a 7700x quadrupole-ICP-MS system (Agilent, Santa Clara, USA). Operating conditions were daily optimized to maximize $^{197}Au^{+}$ following the instrument user's guide (Table 1).

On account of the limited volume of sample available in the immunoassay (100 µL), a micronebulizer (OneNeb, Ingeniatrics, Sevilla, Spain) coupled to a double pass quartz spray chamber (Agilent, Santa Clara, USA) was selected as the sample introduction system. Using this configuration, $^{197}Au^{+}$ sensitivity for a sample uptake rate of 0.6 mL min^{-1} were 3-fold higher than those obtained using the standard nebulizer provided with the instrument (i.e., Micromist, Glass Expansion, Australia). These results were the expected taking into account the higher aerosol generation efficiency of the former nebulizer when operating at sample uptake rates in the order of µL min^{-1} (Almagro, Gañán-Calvo, Hidalgo, & Canals, 2006). Initially, samples were tried to introduce into the spectrometer using self-aspirating conditions (i.e., without using a peristaltic-pump) as suggested by Giesen et al. (2010). However, signal reproducibility was poor (relative standard deviation, RSD, in the 5-8% range) and strong Au memory effects were registered. In fact, wash-out times (defined as the time required for reaching the 1% of the stable signal after blank introduction) of around 300 s for 1 µg Au L^{-1} were obtained. This behavior could be attributed to the Au surface sticky nature (Giesen, Waentig, Panne, & Jakubowski, 2012) and the low sample uptake rate used (Mora, Maestre, Hernandis, & Todolí, 2003). To improve the reproducibility and the sample throughput, a flow injection analysis (FIA) procedure was employed. In this operating mode, samples were introduced into a carrier solution controlled by a peristaltic pump (Model Minipulse 3, Gilson, France) with the aid of a V-451 flow injection manifold (Upchurch Scientific, Silsden, United Kingdom) equipped with a 75 µL loop valve and a syringe. Carrier flow rate was set at 0.6 mL min^{-1} for high throughput analysis. To minimize Au wash-out times, different carrier solutions

(i.e. water, diluted nitric acid, etc.) were tested. Finally, a 1% V V^{-1} hydrochloric acid and 1% w V^{-1} thiourea mixture was chosen since it provides the lowest wash-out times (i.e., lower than 40 s) (Chen, Wee, & Brindle, 2000). Nonetheless, further improvement was feasible spiking the digestion acid mixture for Au nanoparticles with 1% w V^{-1} thiourea. Operating this way, no differences on wash-out times between Au and other elements (e.g. Mn, Ir, etc.) were observed (25 s). Finally, because of the discontinuous sample introduction mode of the FIA device, a peak-shape signal is obtained. Microsoft Excel software was employed to integrate $^{197}Au^{+}$ signals manually.

2.5. Calibration

AFM1 determination was performed by means of a calibration curve built with the $^{197}Au^{+}$ ICP-MS signal response of AFM1 standards of concentrations ranging from 0.001 to 5 µg kg^{-1}. To improve accuracy and precision, Ir signal ($^{193}Ir^{+}$) was employed as internal standard for Au measurements. Ionization potential and m/z values for Ir are closed to the Au ones and, hence, matrix and drifts effects are expected to be similar for both elements (Vanhaecke, Vanhoe, Dams, & Vandecasteele, 1992). Therefore, the $^{197}Au^{+}$ and $^{193}Ir^{+}$ signal ratio was really employed to build the calibration curve and quantify AFM1. Iridium was added to the standards and the unknown samples with the acid mixture employed to digest Au nanoparticles after the immunoassay procedure (section 2.3.) for a final concentration of 2.5 µg L^{-1}.

Finally, it is important to remark that, unlike the conventional ICP-MS analysis, the use of a competitive immunoassay makes that a high ICP-MS signal is related to a low AFM1 concentration. Thus, for instance, when no AFM1 is

present in the sample, all the primary antibody is retained in the microtiter plate and, as a consequence, the Au signal is maximum. Because of the sigmoidal curve response of the competitive immunoassay procedure, analyte determination was circumscribed to the curve section where there was a lineal relationship between the analyte signal and AFM1 concentration logarithm (i.e. 0.012-2.5 µg kg^{-1} under optimum assay conditions).

2.6. Samples

The AFM1 certified reference material of whole milk powder, ERM-BD284 (certified at 0.44±0.06 µg kg^{-1}), was purchased from the Institute for Reference Materials and Measurements (European Commission Joint Research Centre, Geel, Belgium). In addition, five cow milk samples (i.e. raw, pasteurized and ultrahigh temperature pasteurized) were obtained from retail markets and supermarkets, stored at 4 ºC and analyzed before their respective expiration dates.

2.7. Sample preparation

The ERM-BD284 milk powder (10.0 g) was suspended in 50 mL of ultrapure water previously heated up to 50 °C using a stirring rod until a homogeneous mixture was obtained. After that, the solution was cooled and then diluted to 100 mL using ultrapure water. So, AFM1 concentration in the reconstituted milk will be of 0.044 µg L^{-1}.

Both the certified and the commercial milk samples were pre-treated before the immunoassay using the procedure described by Huang et al. (2014) with some minor modifications. Thus, 200 µL of milk were mixed with 800 µL of acetonitrile

in an Eppendorf tube to extract AFM1 and remove matrix components. The extraction was performed using a vortex mixer for 2 min and sonicating the mixture for 30 min (Vibramix, J.P. Selecta S.A., Barcelona, Spain). Then, the extracts were centrifuged at 12100 g for 10 min at 4ºC (A 5804R Centrifuge, Eppendorf, Hamburg, Germany). After that, 800 µL of the supernatant were collected and evaporated up to dryness (miVac Quattro concentrator, Genevac Ltd, Suffolk, UK). The residue was reconstituted with 100 µL of PBS and then analyzed.

3. Results

3.1. Immunoassay optimization

The competitive immunoassay described in the present paper has been developed with a polyclonal antibody produced in rabbits by immunization with BSA haptenized with AFM1. Considering that not only may this IgG preparation contain antibodies which react with BSA but also bovine milk contains a significant amount of this compound (≈1.2 % w V^{-1}), an excess of BSA (1% w V^{-1}) was included in all the incubation media (see section 2.2) in order to neutralize any antibody activity specific to BSA in the assay. According to the supplier, the anti-AFM1 primary antibody is highly specific for AFM1 determination with low cross-reactivity against other aflatoxins (AFB1 2%; aflatoxin B2 0.4%; aflatoxin G1 0.4% and aflatoxin G2 0.1%).

Variables selected for the immunoassay optimization were the concentration of: (i) AFM1-BSA conjugate; (ii) anti-AFM1 primary antibody; and (iii) Strep-Au. The concentration of the secondary biotinylated antibody was not optimized and the dilution factor recommended by the manufacturer (1:2000) was employed. The

optimal conditions for the inhibition assay were chosen by checkerboard titration experiments as described for ELISA (Gee, Miyamoto, Goodrow, Buster, & Hammock,1988; Crowter, 2009). Briefly, decreasing amounts of M1 antigen (AFM1-BSA) were bound to microtiter wells and then incubated with serial dilutions of the primary antibody. ICP-MS readouts were evaluated to identify the optimal amount of AFM1-BSA per well as well as the corresponding dilution of primary antibody. Primary antibody was prepared by serial dilutions from 1:500 to 1:8000 whereas AFM1-BSA concentration was modified between 0.07 and 10 ng mL^{-1}. For these experiments, Strep-Au dilution factor was kept constant at 1:500. In titration experiments, optimal conditions were defined as the amount of reagents (both, AFM1-BSA and primary antibody) producing signal-to-background ratio in ICP-MS close to the 80% of the maximal signal at plateau, characteristic of an antibody excess conditions (Crowter, 2009). The rationale for this criterion was that an assay dependent on inhibition of antibody binding, sensitivity would be maximal at the lowest concentration of antibody producing a consistent readout (i.e. minimal variability in replicates and an adequate dose-response relationship, when tested with the lowest possible amount of BSA-M1 bound to solid phase). Experimental results showed that optimum response was obtained for 0.35 ng L^{-1} of AFM1-BSA and a primary antibody dilution factor of 1:2000 (see Table S1, supplementary material). Once the optimum AFM1-BSA and primary antibody concentration were selected, Strep-Au dilution factor was optimized following a similar procedure to ensure the maximum $^{197}Au^{+}$ response by the mass spectrometer. To this end, this parameter was modified between 1:1000 and 1:62.5. As expected, when the Strep-Au dilution factor was decreased, the Au response in ICP-MS improved.

A 6-fold signal improvement was registered when the dilution factor was varied from 1:1000 to 1:62.5 (see Figure S1, supplementary material). On the other hand, the precision in ICP-MS was also observed to be dependent on the Strep-Au dilution employed. Gold signal RSD was improved up to 2-3% when Strep-Au dilution decreased down 1:250. No further improvement of the RSD was obtained when lower dilution factors were used. Though lower Strep-Au dilutions provide higher Au signals in ICP-MS, no real improvement on analytical figures of merit was obtained (this topic will be discussed in detail below). For this reason, 1:250 Strep-Au dilution was selected for further studies as a compromise between analytical performance and costs.

For calibration purposes, AFM1 standards were treated under the optimum competitive immunoassay experimental conditions (Figure 2). As it has been described in the experimental section, the sample containing the AFM1 and the primary antibody solution were incubated together (Figure 1, step 1.b) and then the mixture was transferred to the microtiter plate for a second incubation step (Fig1, step 2). Initially, the incubation time of each step was 1 hour. Analytical response in Figure 2 is expressed as the inhibition factor, defined as $(S_0-S)\cdot 100/S_0$ where S_0 is the maximal signal obtained in wells with no inhibition (i.e. no AFM1 was added) and S is the signal observed for each sample or standard preparation. Figure 2 shows that the concentration of AFM1 giving rise to a 50% inhibition factor (i.e., half maximal inhibitory concentration, IC_{50}) for the ICP-MS-based immunoassay was 6.4 µg kg^{-1} whereas LoD (calculated as three times the standard deviation of the signal of 15 blank replicates (Jiang, Wang, Nolke, Zhang, Niu, & Shen, 2013) was around 0.070 µg kg^{-1}, low enough to analyze AFM1 according to USA legislation ($AFM1_{max\ level}$: 0.500 µg kg^{-1}).

However, unless a pre-concentration step would be implemented, the immunoassay could not be applied to the analysis of milk samples ($AFM1_{max\ level}$: 0.050 µg kg^{-1}) and infant formula ($AFM1_{max\ level}$: 0.025 µg kg^{-1}) under the more restrictive EU policy. In order to reduce the minimum AFM1 concentration level detectable and avoid costly and long sample pretreatments based on immunocolums for AFM1 preconcentration (Dragacci, Grosso, & Gilbert, 2001; Beltran, Ibañez, Sancho, Cortés, Yusa, & Hernández, 2011) the immunoassay optimization procedure was revised.

Competitive immunoassay LoD is strongly linked to both primary antibody dilution factor and detector capability to recognize the primary antibody retained in the microtiter plate after the incubation step with the analyte containing sample. Thus, primary antibody dilution factor could be decreased to make the immunoassay more sensitive to low AFM1 levels. However, this means that primary antibody retained in the microtiter plate is less significant, thus making detection more difficult or even impossible. Accordingly, LoDs for the ICP-based immunoassay are expected to improve by increasing the efficiency of primary antibody retention in the microtiter plate and/or detection by ICP-MS. First of all, the incubation time of the AFM1 and the anti-AFM1 primary antibody in the microtiter plate coated with AFM1-BSA (step 2) was increased from 1 to 2.5 hours with the aim to favor primary antibody retention on it. Operating in this way, it was feasible to reduce primary antibody dilution factor from 1:2000 to 1:4000, thus improving sensitivity and LoDS without compromising robustness. As a consequence, the IC_{50} was approximately 10 times lower (0.42 µg kg^{-1}) and LoD decreased down to 0.005 µg kg^{-1} (Figure 2). The use of higher incubation times together lower primary antibody dilution factor (<1:4000) was

not further explored since sample throughput is negatively affected and LoD were low enough to quantify AFM1 according to the current international policies for this analyte. Alternatively, the feasibility of using lower Strep-Au dilution factor to improve ICP-MS detection was checked but no real improvement was obtained on LoDs. It should be taking into account that secondary antibody has a limited amount of biotin moieties and, as a consequence, signal amplification with Strep-Au is limited and could not compensate the lower amount of primary antibody retained in the microtiter plate. Probably, LoD could be improved using a sector field instrument or with a heteroatom-tag with a higher sensitivity in ICP-MS in order to use lower primary antibody dilution factors.

3.2 Method validation

The analytical methodology was evaluated according to European Conformity guidelines for analytical methods of food contaminants and mycotoxins (CE, 2002; CE, 2006). First of all, method accuracy and precision was checked by analyzing an AFM1 certified reference material (ERM-BD284 milk powder, 0.044±0.006 µg kg^{-1}) and different milk samples spiked with known amounts of AFM1. Recovery test was performed spiking milk samples with AFM1 standard for a final concentration of 0.030 and 0.080 µg kg^{-1}. All the samples were analyzed following the optimized immunoassay after an extraction treatment with acetonitrile to mitigate the effects of matrix components (e.g. proteins, fats etc.) on the antibody reaction. In fact, direct analysis of milk samples produced systematically AFM1 recovery values between 200-300%. Sample dilution to mitigate matrix effects was not explored due to its negative impact on LoDs.

Table 2 shows the recovery values for AFM1 in the certified reference material and in the spiked milk samples after the sample pre-treatment with acetonitrile. There is observed to be a good agreement between experimental and theoretical values. AFM1 recovery values ranged between 80% and 102%. These values were within the limits established by the EU for analyte concentrations below 1 µg kg^{-1} (-40%/+20%). The repeatability (intra-assay precision) of the method was determined by analyzing five replicates of each sample on the same day. The RSD of the AFM1 concentration levels was in the 5%15% range. These values are the typical for immunoassays. The immunoassay reproducibility (inter-assay precision) of the proposed methodology was evaluated as the RSD of the measurements obtained for five independent immunoassays performed in five different days. The average AFM1 concentration obtained for the certified reference material was 0.042±0.008 µg kg^{-1} which highlights the reproducibility of the assay. Similar conclusions were obtained for the spiked milk samples. Finally, the lower and the upper quantification limit of the immunoassay (LLOQ and ULOQ) were estimated (Guidance for Industry, Bioanalytical Method Validation, FDA, 2001). The LLOQ was defined as the analyte concentration that has a response at least 3 times that of a blank sample and repeatability lower than 20%. Similarly, the ULOQ was defined as the highest concentration standard that signal response has repeatability lower than 20%. LLOQ and ULOQ experimental values were 0.012 µg kg^{-1} and 2.5 µg kg^{-1}, respectively. These results demonstrate the suitability of the ICP-MS-based immunoassay for AFM1 determination in milk samples according to current security policies for this analyte. In fact, this methodology was applied to the analysis of different milk

samples (i.e. raw, pasteurized or ultra-high temperature pasteurized) but no AFM1 was detected (<0.005 μg kg^{-1}).

3.3 Comparison with other methodologies

Analytical figures of merit of the ICP-MS-based immunoassay have been compared with those previously reported in the literature (Table 3). In general, the methodology developed in this work affords similar results to those obtained with chromatographic-based approaches or even other immunoassays. When comparing to chromatographic methods, poorer precisions are obtained (RSD nearly 2 to 3-fold higher), but no laborious sample pretreatments based on solid phase extraction or immunocolumns are required to preconcentrate and purify the AFM1. Sample pretreatment proposed in the present work is quite simple and it is only focused to eliminate the most significant matrix components without compromising sample throughput. Nevertheless, a 2-fold AFM1 preconcentration factor is obtained operating on this way. On the other hand, due to immunoassay simplicity, reagents and solvent requirements (as well as wastes) are minimized. As regards other immunoassay procedures, ICP-MS-based detection offers a wider linear range. Moreover, the proposed method not only shows lower background and blank levels but also an independent analytical response from incubation and storages times. In fact, standards and samples in the microtiter plate can be stored 2-3 weeks after acid mixture addition without significant effects on AFM1 results. However, sample throughput is partially degraded since microtiter plate wells are sequentially analyzed by the ICP-MS (3-4 hours to analyze a 96-well plate).

Up to date, the use of ICP-MS as a detector for mycotoxin analysis has been limited. In fact, it has only previously used to quantify ochratoxin A levels in wine by means of a competitive immunoassay methodology (Giesen, Jakubowski, Panne, & Weller, 2010). Though this previous work is not focused on AFM1, it is worth to compare both methodologies due to their similarities. Thus, Ochratoxin A detection was accomplished using secondary antibodies functionalized with Au nanoparticles and a sector field based ICP-MS. LoD using this configuration was stablished at 0.003 µg kg^{-1}. A priori the high detection capabilities found could be attributed (at least in part) to the high sensitivities afforded by sector field mass spectrometers which makes feasible to use a low primary antibody dilution factor (1:10000). However, our work shows that AFM1 can also be quantified in the ng kg^{-1} range using a quadrupole-based mass spectrometer with a two-step signal amplification procedure based on a secondary biotinylated antibody and Strep-Au. The possibility of using quadrupole-based ICP-MS instruments to quantify toxin at ultra-trace levels is advantageous since they are the most spread mass spectrometers worldwide. Furthermore, precision for the AFM1 immunoassay was significantly better (5-15%) than that for the Ochratoxin A (5-40%). The origin of these differences is not clear due to the different procedures implemented in each immunoassay. Nonetheless, it points out that the precision achievable for the ICP-MS-based immunoassay is mainly limited by the immunological step since uncertainty derived by the ICP-MS lays below 5%.

4. Conclusions

This work demonstrates that ultra-trace analysis of AFM1 in milk is feasible using an ICP-MS-based immunoassay. Analytical figures of merit of this method fulfil the most restrictive current international policies for AFM1 analysis in milk and dairy products. Despite the fact that sample throughput could be deteriorated in relation to other immunoassay methodologies described in the literature, the use of ICP-MS for mycotoxin analysis has a great potential in food analysis due to a higher dynamic range, lower background levels and the independence of analytical response from incubation or storage times. In this regard, aflatoxin detection by means of ICP-MS could be still improved. Thus, LoDs for the competitive immunoassay employed in this work strongly depend on the primary antibody dilution factor and ICP-MS Au sensitivity to detect the amount of primary antibody retained in the microtiter plater. Therefore, detection capabilities could be probably improved (below ng L^{-1}) using lower primary antibody dilution factor with a sector field mass spectrometer and/or tagging with higher sensitive hetereoatoms in ICP-MS. On the other hand, methodology sample throughput could be enhanced significantly determining other mycotoxins together AFM1 by means of antibodies functionalized with different heteroatoms. Operating on this way ICP-MS multiplexing capabilities are exploited and the use of a mass spectrometer could be more beneficial than other immunoassay detection procedures (e.g. ELISA, etc.). These experiments are currently being carried out in our laboratories.

Acknowledgments

The authors would like to thank the Generalitat Valenciana (Project GV/2014/138) and the Vice-Presidency for Research of the University of Alicante (Project GRE12-19) for the financial support of this work.

All the manuscript authors declare that there is not any actual or potential conflict of interest including any financial, personal or other relationships with other people or organizations that could inappropriately influence, or be perceived to influence, the results presented in the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:...'.

References

Aguilera-Luiz, M.M., Plaza-Bolaños, P., Romero-González, R., Martínez Vidal, J.L. & Garrido Frenich, A. (2011). Comparison of the efficiency of different extraction methods for the simultaneous determination of mycotoxins and pesticides in milk samples by ultra high-performance liquid chromatography-tandem mass spectrometry. *Analytical Bionalytical Chemistry, 399*, 2863-2875.

Almagro, B., Gañán-Calvo, A.F., Hidalgo, M. & Canals, A. (2006) Flow focusing pneumatic nebulizer in comparison with several micronebulizers in inductively coupled plasma atomic emission spectrometry. *Journal of Analytical Atomic Spectrometry, 21*, 770-777.

Bettmer, J., Montes-Bayón, M., Ruiz Encinar, J., Fernádez, M.L., Fernández de la Campa, M. & Sanz-Medel, A. (2009). The emerging role of ICP-MS proteomics analysis. *Journal of proteomics, 72*, 989-1005.

Bhat, R., Rai, R.V., & Karim A. A. (2010). Mycotoxins in food and feed: present status and future concerns. *Comprehensive reviews in food science and food safety, 9*, 57-81.

Beltran, E., Ibañez, M., Sancho, J.V., Cortés, M. A., Yusa, V. & Hernández, F. (2011). UHPLC-MS/Ms highly sensitive determination of aflatoxin, the aflatoxin metabolite M1 and ochratoxin A in baby food and milk. *Food Chemistry, 126,* 737-744.

Chen, W., Wee, P. & Brindle, I.D. (2000). Elimination of the memory effects of gold, mercury and silver in inductively coupled plasma atomic emission spectroscopy. *Journal of Analytical Atomic Spectrometry, 15*, 409-413.

Crowter J.R. (2001) The ELISA Guidebook. (2nd ed.) New Jersey, Humana press.

Diniz Andrade, P., Laine Gomez da Silva, J. & Dutra Caldas, E. (2013). Simultaneous analysis of afaltoxins B1, B2, G1, G2, M1 and ochratoxin A in brest milk by high-performance liquid chromatography/fluorescende after liquid-liquid extraction with low temperature purification (LLE-LTP). *Journal of Chromatography A, 1304*, 61-68.

Dragacci, S., Grosso, F. & Gilbert, J. (2001). Immunoaffinity column cleanup with liquid chromatography for determination of Alfatoxin M1 in liquid milk: collaborative study. *Journal of AOAC International, 84,* 437-443.

EC (2001). Commission Regulation, Setting Maximum Levels for Certain Contaminants in Food Stuffs, *Official Journal of the European Union L, 077*, 1–13.

EC (2002). Commission Decision 2002/657/EC of 12 August 2002 implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results, as amended by Decision 2003/181/EC (4). *Official Journal of the European Communities*, 8–36.

EC (2004). Commission Regulation, Regards Aflatoxins and Ochratoxin a in Foods for Infants and Young Children, *Official Journal of the European Union L, 106*, 5-24.

EC (2006). Commission Regulation (EC) No. 1881/2006 of 19 December 2006 setting maximum levels for certain contaminants in foodstuffs. *Official Journal of the European Union L, 364*, 5–24.

FDA, Sec. 527.400 Whole milk, Low fat milk, skim milk – aflatoxin M1 (CPG 7106.10), FDA/ORA Compliancy Guides 2005.

Flor-Flores, M.E., Lizarraga, E., López de Cerain, A. & González-Peñas, E. (2015). Presence of mycotoxins in animal milk: a review. *Food Control, 53*, 163-176.

Pico, Y. (2007) Food Toxicants Analysis. (1st ed). Amsterdam, Elsevier, (chapter 5)

Giesen, C., Jakubowski, N., Panne, U. & Weller, M.G. (2010). Comparison of ICPMs and photometric detection of an immunoassay for the determination of ochratoxin A in wine, *Journal of Analytical Atomic Spectrometry, 25*, 1567-1572.

Giesen, C., Waentig, L., Panne, U. & Jakubowski, N. (2012). History of inductively coupled plasma mass spectrometry-based immunoassays. *Spectrochimica. Acta Part B, 76*, 27-39.

Gee, S.J., Miyamoto, T., Goodrow, M.H., Buster, D. & Hammock, B.D. (1988). Development of an enzyme-linked immunosorbent assay for the analysis of the thiocarbamate herbicide molinate. *Journal of Agricultural and Food Chemistry, 36,* 863–870.

Guan, D., Li, P., Zhang, W., Zhang, D. & Jiang, J. (2011). An ultra-sensitive monoclonal antibody-based competitive enzyme immunoassay for aflatoxin M1 in milk and infant milk products, *Food Chemistry 125, 1359-1364*

Guidance for Industry, Bioanalytical Method Validation, US Department of Health and Human Services, Food and Drug Administration Centre for

Drug Evaluation and Research (CDER), Centre for Veterinary Medicine (CVM), May 2001.

Gurbay, A., Aydin, S., Girgin, G., Engin, A.B. & Sahin, G. (2006). Assessment of aflatoxin M1 levels in milk in Anakara, Turkey. *Food Control, 17*, 1-4.

Hashemi, M. & Taherimaslak, Z. (2014). Determination of aflatoxin M1 in liquid milk using high performance liquid chromatography with fluorescence detection after magnetic solid phase extraction. *RSD Advances, 4*, 33497-33506.

Huang, L.C., Zheng, N., Zheng, B.Q., Wen, F., Cheng, J.B., Han, R.W., Xu, X.M., Li, S.L. & Wang, J.Q. (2014). Simultaneous determination of aflatoxin M1, ochratoxin A, zearalenone and α-zearalenol in milk by UHPLC–MS/MS. *Food Chemistry, 146*, 242–249.

International Agency for Research on Cancer. Monographs on the evaluation of carcinogenic risks to humans. Some traditional herbal medicines, some mycotoxins, naphthalene and styrene, World Health Organization (WHO), Lyon, France, 2002, vol. 82, 171-174.

Jiang, W., Wang, Z., Nolke, G., Zhang, J., Niu, L. & Shen, J. (2013). Simultaneous determination of aflatoxin B1 and aflatoxin M1 in food matrices by enzyme-linked immunosorbent assay. *Food Analytical Methods, 6*, 767-774.

Kretschy, D., Koellensperger, G. & Hann, S. (2012). Elemental labelling combined with liquid chromatography inductively coupled plasma mass spectrometry for quantitation of biomolecules: A review. *Analytica Chimica Acta 750*, 98-110.

Liu, R., Wu, P., Yang, L., Hou, X. & Lv, Y. (2014). Inductively coupled plasma mass spectrometry-based immunoassay: a review. *Mass Spectrometry Reviews, 33*, 373-393.

Montoro Bustos, A.R., Trapiella-Alfonso, L., Ruiz Encina, J., Costa-Ferández, J.M., Pereiro, R. & Sanz-Medel, A. (2012). Elemental and molecular detection for quantum dots-based immunoassays: a critical appraisal. *Biosensors and bioelectronics, 33*, 165-171.

Mora, J., Maestre, S., Hernandis, V. & Todolí, J.L. (2003). Liquid-sample introduction in plasma spectrometry. *Trends in Analytical Chemistry, 22*, 123-132.

NIST atomic spectradatabase (version 5), http://physics.nist.gov/cgi-bin/ASD/ie.pl. Accessed 01.12.16.

Pei, S.C., Zhang, Y.Y., Eremin, S.A. & Lee, W.J. (2009). Detection of aflatoxin M1 in milk products from China by ELISA using monoclonal antibodies. *Food Control, 20*, 1080–1085.

Prandini, A., Tansini, g., Sigolo, S., Filippi, L., Laporta, M. & Piva, G. (2009). On the occurrence of aflatoxin M1 in milk and dairy products. *Food and Chemical Toxicology: an international journal published for the British industrial biological research association, 47*, 984-991.

Radoi, A., Targa, M., Prieto-Simon, B. & Marty, J.L. (2008). Enzyme-Linked Immunosorbent Assay (ELISA) based on superparamagnetic nanoparticles for aflatoxin M1 detection. *Talanta, 77*, 138–143.

Reiter, E., Zentek, J. & Razzazi, E. (2009). Review of sample preparation and methods used for the analysis of aflatoxins in food and feed. *Molecular Nutrition Food Research.* 53, 508-524.

Shephard, G.S. (2008). Determination of mycotoxoins in human foods. *Chemical Society Reviews,* 37, 2468-2477.

Sørensen, L. K. & Elæk, T.H. (2005). Determination of mycotoxins in bovine milk by liquid chromatography tandem mass spectrometry. *Journal of Chromatography B, 820*,183–196

Tholey, A. & Schaumlöffel, D. (2009). Metal labeling for quantitative protein and proteome analysis using inductively-coupled plasma mass spectrometry. *Trends in Analytical Chemistry, 29*, 399-408.

Vanhaecke, F., Vanhoe, H., Dams, R. & Vandecasteele, C. (1992). The use of internal standards in ICP-MS. *Talanta, 39*, 737-742.

Vdovenko, M.M., Lu, C.C., Yu, F.Y. & Sakharov, I.Y. (2014). Development of ultrasensitive direct chemiluminescent enzyme immunoassay for determination of aflatoxin M1 in milk. *Food Chemistry, 158,* 310-314.

Figure 1. Scheme of the competitive ICP-MS-based immunoassay for AFM1 analysis in milk samples.

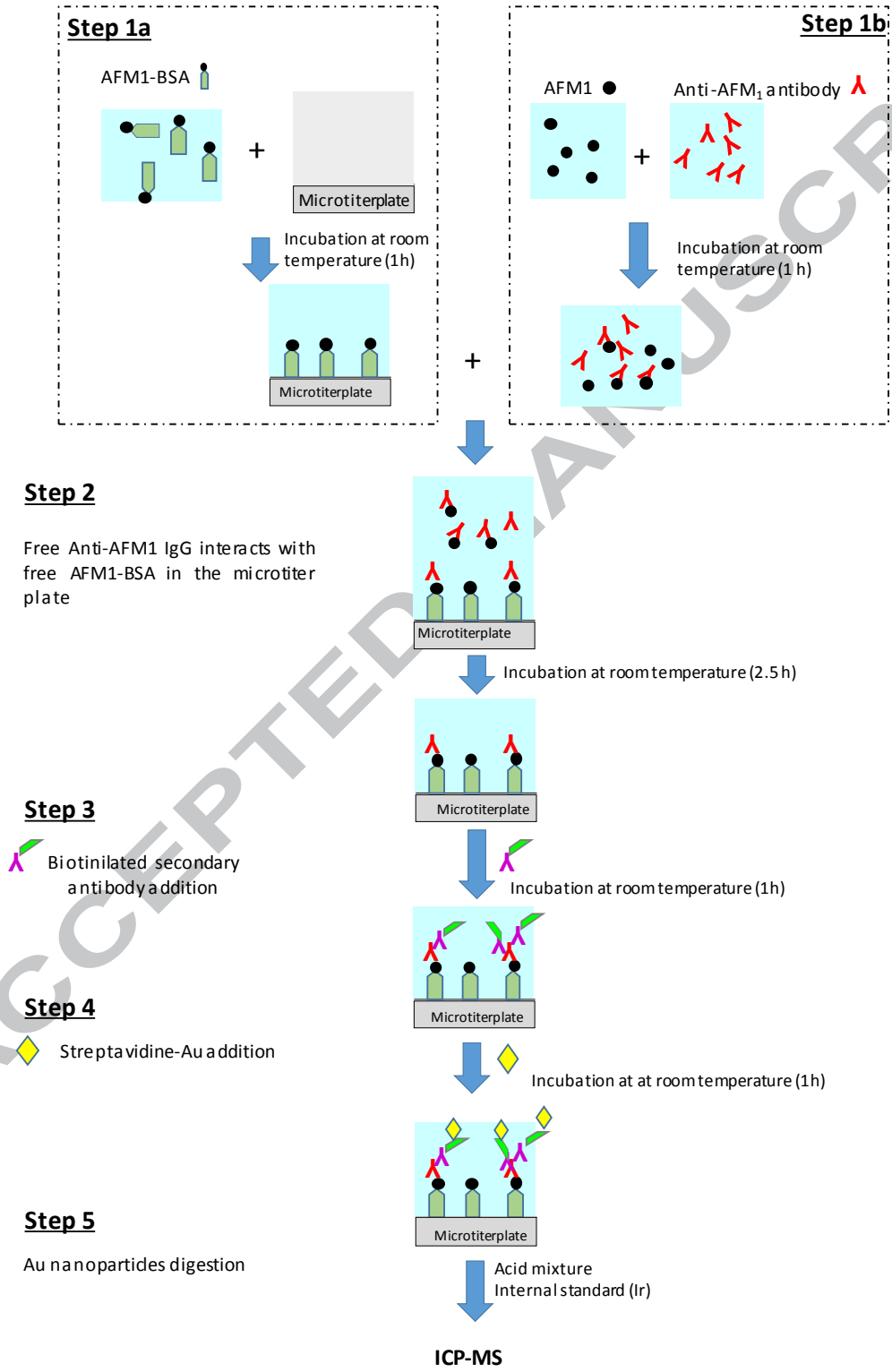

Figure 2. AFM1 calibration curve using different primary anti-AFM1 antibody concentrations. (-▲-) primary antibody 1:2000/incubation time 1h; (-●-) primary antibody 1:4000/incubation time 2.5 h. AFM1-BSA concentration: 0.35 ng mL^{-1}; secondary antibody dilution factor: 1:2000; Strep-Au dilution factor: 1:250.

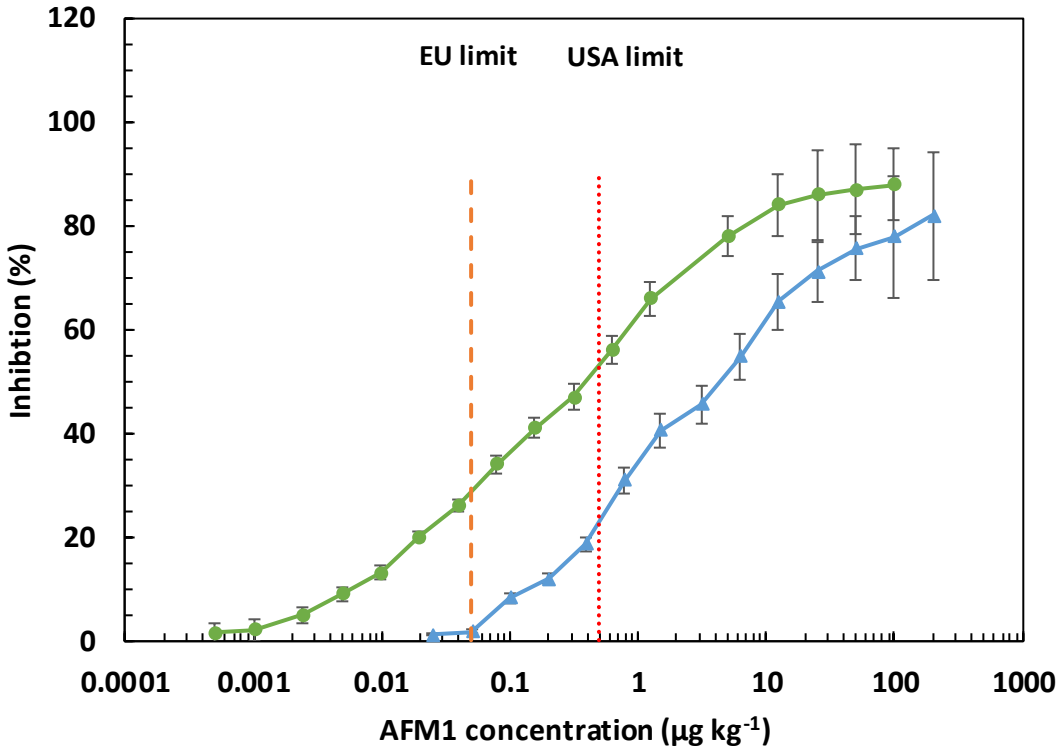

Table 1. Operating conditions employed in ICP-MS

Agilent 7700x ICP-MS	
Plasma forward power (W)	1550
Argon flow rate (L min^{-1}):	
Plasma	15
Auxiliary	0.9
Nebulizer	1.01
Sample introduction system	
Nebulizer	OneNeb micronebulizer
Spray chamber	Double pass
Carrier flow rate (mL min^{-1})	0.6
Dwell time (µs)	0.5
Number of sweeps	100
Replicates	90
Signal nature	Area

Table 2. AFM1 recovery assay for different kind of milk samples.

	AFM1 concentration (μg kg^{-1})		Recovery (%)*
Sample	**Certified/Spike**	**Experimental**	
Certified milk powder	0.044±0.006	0.038±0.006	87±10
Raw milk	0.030	0.027±0.003	90±10
	0.080	0.078±0.004	93±5
Pasteurized milk	0.030	0.024±0.005	80±17
	0.080	0.078±0.002	98±2
UHT whole milk	0.030	0.026±0.003	87±8
	0.080	0.079±0.010	99±10
UHT whole milk (2)	0.030	0.028±0.003	93±7
	0.080	0.082±0.008	102±10

Replicates: 5, * mean ± standard deviation

Table 3. Comparison of diverse analytical methodologies proposed in the literature for the determination of AFM1 in milk samples

Methodology	Recovery (%)	Precision (%)	LOD (μg kg^{-1})	Linear range (μg kg^{-1})	Reference
Indirect competitive immunoassay (ICP-MS)	80-102	5-15	0.005	0.010-2.5	This work
Liquid-liquid extraction HPLC-Fluorescence	73-99	2-7	0.05	-	Diniz Andrade, Laine Gomez da Silva, & Dutra Caldas, 2013
IAC-HPLC-FD	116	5	0.010	0.01-0.20	Gurbay, Aydin, Girgin, Engin, & Sahin, 2006
MSFE-HPLC-FD	91-102	5	0.005	0.015-10	Hashemi, & Taherimaslak, 2014
SPE-LC-MS	78-108	5-10	0.010	0.020-1	Sørensen, & Elæk, 2005
UPHLC-MS/MS	84-97	13	0.010	-	Aguilera-Luiz, Plaza-Bolaños, Romero-González, Martínez Vidal, & Garrido Frenich, 2011
	80-110	<10	0.005	0.025-10	Beltran, Ibañez, Sancho, Cortés, Yusa, & Hernández, 2011
Indirect competitive immunoassay (ELISA)	80-102	5-17	0.040	0.040-0.500	Pei, Zhang, Eremin, & Lee, 2009
Direct competitive ELISA	90-110	<10	0.003	-	Guan, Li, Zhang, Zhang, & Jiang, 2011
Direct competitive ELISA	93-98	5-8	0.008	0.004-0.250	Radoi, Targa, Prieto-Simon, & Marty, 2008

Highlights

- A novel ICP-MS-based immunoassay method to quantify AFM1 in milk samples is presented
- AFM1 detection is based on a competitive immunoassay using gold nanoparticles
- Limits of detection fulfill international policies for Aflatoxin M1